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A Genetically Diabetic Model "KK-CA^y Mice" for a Pharmacological Assay

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Synopsis

A genetically diabetic model, KK-CA^y mice which were bred by mating female KK mice (aa, BB, cc) with male KK-CA^y mice (A^{y}a, BB, CC) was studied on the usefulness as a tool for a pharmacological assay. Body weights of KK-CA^y mice increased more rapidly than those of control mice, KK-C. When the body weights of male KK-CA^y mice reached about 30 g 10 weeks after birth, their blood glucose levels increased. Severe hyperglycemia (over 300 mg/100 ml) was often observed in the males, but not in the females. Glucose tolerance in the KK-CA^y mice was more markedly impaired than that in the control mice. The increase in blood FFA level correlated with the increase in body weight on both KK-CA^y mice and the controls. On hyperinsulinemia observed, the ratio of plasma immunoreactive insulin (IRI) level to blood glucose level in the male mice was lower than that seen in the female mice. On hyperglucagonemia observed, elevation of plasma immunoreactive glucagon (IRG) was more remarkable in the males than in the females. Morphological study showed insular degranulation only in the males. Since the dose-dependent insulin-induced falling was observed on blood glucose level in nonfasted KK-CA^y mice, they could be used as a feasible tool for an assay of antidiabetic drugs.

Much effort has been directed towards developing suitable experimental models in the investigation of etiology, therapy, and genetics of diabetes mellitus. Genetically diabetic animals KK mice have attracted attention since the first studies on them were reported by Nakamura (1962). Nishimura (1969) transferred the yellow obese gene (A^y) into KK mice to breed mice strain "KK-CA^y" for severe diabetic syndrome. Iwatsuka *et al.* (1970) and Shino and Iwatsuka (1970) have already reported the general features and the morphological observations on "Yellow KK mice".

The purpose of the present study is to confirm the physiological and pathological features of KK-CA^y mice and their metabolic abnormalities in order to obtain a possible model of diabetes mellitus as a feasible tool for a pharmacological assay.

Received July 8, 1978.

Materials and Methods

Animals

All the mice used in the present study were derived from the mice colony in our laboratory. KK-CA^y mice were bred by mating female KK mice (aa, BB, cc) with male KK-CA^y mice (A^{y}a, BB, CC) which were obtained from Biological Research Laboratory of Takeda Chemical Industries, as shown in Fig. 1. Fig. 2 shows the breeding schedules of KK-CA^y mice. Offsprings of the breeders were classified into four genotype groups and three phenotype groups (Fig. 2-A). KK-CA^y mice were efficiently obtained in the ratio of one-half by the schedules shown in Fig. 2-B. The littermate KK-C mice (aa, BB, Cc) were used as controls in the present study (Fig. 2-C). Mice were maintained

under the constant temperature (23±1°C) and fed on the usual laboratory diet (CA-1, Japan Clea Inc., Tokyo) and tap water freely.

Glucose tolerance test and insulin application

Mice fasted for 5 hrs were loaded glucose of 2 g/kg in 20% solution intraperitoneally twice. Nonfasted mice were given intraperitoneally 0.1 ml/10 g body weight of insulin solution.

Chemical procedures

Blood samples were obtained from the orbital vein plexus of mice by capillary glass. Blood glucose was estimated by the method of Momose *et al.* (1963). Blood free fatty acids (FFA) were determined by the colorimetric method of Itaya and Ui (1965).

Fig. 1. An appearance of overgrowth in a KK-CA^y mouse (male, yellow coat) compared with a control KK mouse (male, albino coat). A scale is scored every one centimeter.

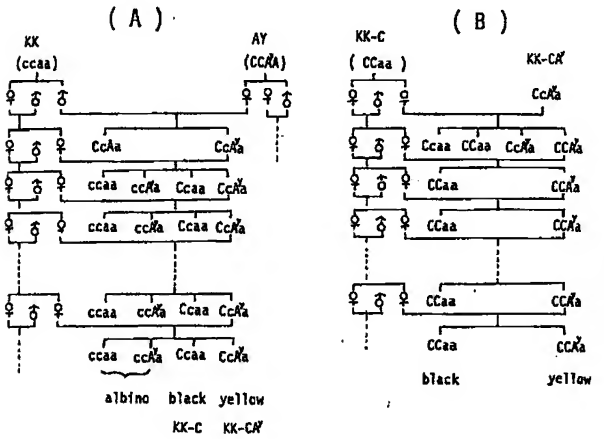

Fig. 2. Cross-intercross system for breeding of KK-CA^y mice. (A) Establishment of a coisogenic (KK-CA^y) strain which has a yellow obese gene (A^y) with genetic background of KK. (B) Efficient breeding of KK-CA^y mice. (C) Offsprings of cross in the present study.

Histological procedures

Tissues were fixed in 10% formalin solution or Bouin's solution. The aldehyde thionin technique (Paget and Eccleston, 1959) was used to stain B-of granules in the islets of pancreas. Liver, kidney, and other tissues were subjected to PAS, AZAN, and hematoxylin-eosin stainings.

Radioimmunoassay of hormones

Plasma insulin and glucagon levels were determined by the modified method of Besbuquois and Aurbach (1971) and Henquin *et al.* (1974), respectively. Bovine insulin standard (Novo) or sample of 100 μl, ^{125}I iodoinsulin 15-25 μCi (Dinabot) of 100 μl, anti-insulin serum, obtained from guinea pig (Hartley, ♂) immunized with bovine insulin, of 200 μl, and borate buffer (pH 8.3) of 400 μl were incubated for 16 hr in 4°C. Porcine glucagon standard (Novo) or sample of 100 μl, ^{125}I-iodoglucagon 1.2-1.8 μCi (Amersham) of 100 μl, anti-glucagon serum K964 (Novo) of 100 μl, and Trasylol 500 KIE (Bayer) in borate buffer (pH 8.3) of 100 μl were incubated for 24 hr in 4°C. Bovine serum of 200 μl was added as a carrier protein in the separation step and the final concentration of polyethylene glycol were 11.5% (IRI assay) and 14.4% (IRG assay), respectively. Radioactivity was determined by Automatic Gamma Counting System, MS-588 (Micromedic System, Inc.).

Results

Correlation between body weight and growth or blood glucose level in KK-CA^y mice

Body weights of most KK-CA^y mice increased more rapidly than those of the control mice (KK-C) and were over 40 g in advancing age (Fig. 3). Fig. 4 shows the correlation between body weight and blood glucose level. Blood glucose level in the male KK-CA^y mice increased after their body weight reached about 30 g at 10 weeks of age, and the severe hyperglycemia (over 300 mg/100 ml) was often observed. No such an evidence, however, was seen in female KK-CA^y mice with body weight below 50 g. The mild hyperglycemia was partly shown in the female mice with body weight over 50 g.

Nonfasted blood glucose level and glucose tolerance in KK-CA^y mice

Instabilty of blood glucose levels in the KK-CA^y mice was observed under the nonfasting condition. Fig. 5 shows frequency distribution of nonfasted blood glucose level in the male KK-CA^y mice and their control littermates at 8 to 30 weeks of age. Nonfasted control mice showed the normal distribution with one peak at about 150 mg/100 ml. Nonfasted KK-CA^y mice showed an irregular pattern of distribution at higher range of concentration. In addition to that their distribution curve extended over a wider range than that of controls, it had two peaks distinguished clearly. The first peak was at about 180 mg/100 ml, and the second peak at around 400–500 mg/100 ml. A glucose tolerance curve in the male KK-CA^y mice was more markedly increased than that in control mice (Fig. 6). In mild hyperglycemic KK-CA^y mice (below 200 mg/100 ml) the blood glucose level in 2 hr after the injection of glucose remained more high than that before the injection. In severe hyperglycemics (over 300 mg/100 ml), this phenomenon was shown more evidently.

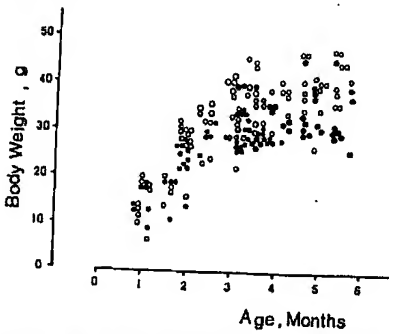

Fig. 3. Correlation between growth and body weight in male KK-CA^y mice (○) and their control littermate KK-C mice (●). Each dot shows one male mouse. The difference of body weight is shown after 2 months of age and the body weight of matured KK-CA^y mice was clearly greater than that of the controls.

Blood FFA levels in KK-CA^y mice

Blood FFA levels in the KK-CA^y mice were compared with those in the control mice on the correlation with increase in body weight. As shown in Fig. 7, their body weight being below 40 g, the blood FFA levels in the KK-CA^y mice increased gradually as well as those in the controls. Their levels increased after their body weights reached about 40 g and were different from those of the controls. The difference between male and female mice was not significantly observed. Fig. 8 shows the blood FFA levels classified by the body weight. The blood FFA level was elevated most significantly in the KK-CA^y mice with doby weight over 40 g, compared with control KK-C mice and alloxan-induced diabetic ddY mice.

Correlation between plasma insulin and blood glucose levels

Plasma immunoreactive insulin (IRI) was determined in both male and female KK-CA^y mice. The IRI levels were markedly

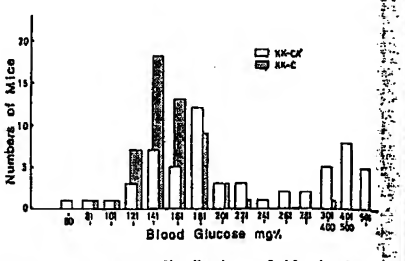

Fig. 5. Frequency distribution of blood glucose level in KK-CA^y mice (□) and control littermates, KK-C (▨) at the age of 8 to 30 weeks. Mice were fed an usual laboratory diet freely. The total numbers of KK-CA^y and KK-C mice used were 59 and 54 respectively.

Fig. 4. Correlation between body weight and blood glucose level in male and female KK-CA^y mice (○) and their control littermate KK-C mice (●), 6–17 weeks of age. Each dot shows one mouse.

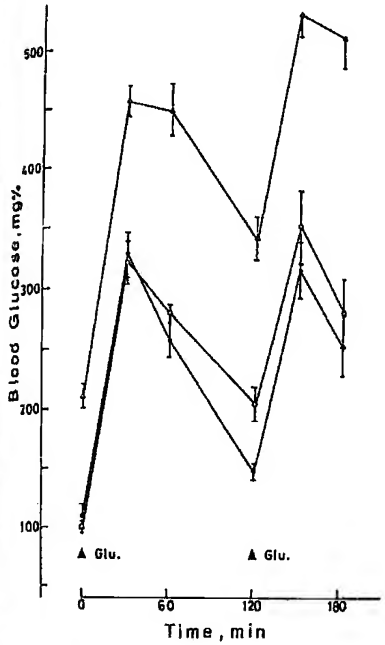

Fig. 6. Double glucose tolerance test in KK-CA^y mice and their control littermate KK-C mice. Mice were fasted for 5 hr and followed by twice glucose (Glu.) injection (2 g/kg ip). Each point of KK-C (●), mild hyperglycemic KK-CA^y (below 200 mg/100 ml, ○), and severe hyperglycemic KK-CA^y (over 300 mg/100 ml, △) represents mean and standard error of 4 to 6 mice.

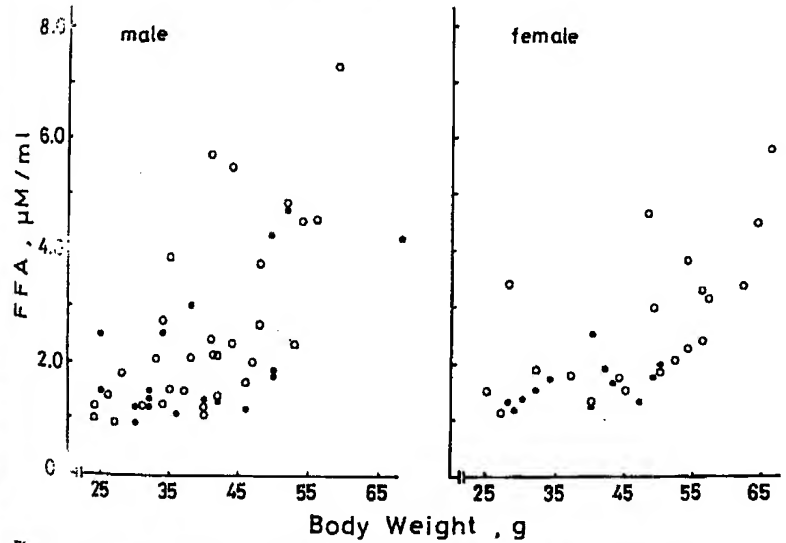

Fig. 7. Correlation between body weight and blood FFA level in male and female KK-CA^y mice (○) and their control littermate KK-C mice (●), 8–30 weeks of age. Each dot shows one mouse.

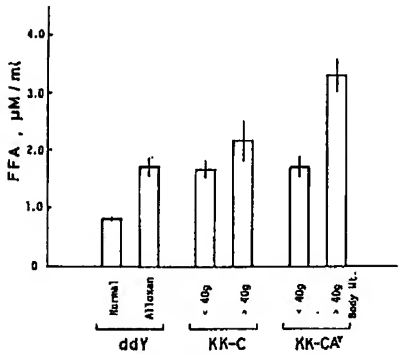

Fig. 8. Blood FFA levels in KK-CA^y and KK-C mice classified by body weight and in alloxan-induced diabetic ddY mice. The column shows mean and standard error of 12 to 31 mice. The alloxan-induced diabetic mice had been given alloxan monohydrate 85 mg/kg iv seven days before FFA determination.

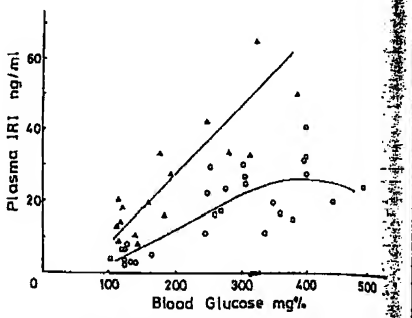

Fig. 9. Correlation between blood glucose level and plasma insulin level in male (○) and female (▲) KK-CA^y mice. Each dot shows one mouse.

Table 1. Plasma levels of glucose, insulin and glucagon in nonfasted KK-CA^y mice and their littermate KK-C mice (10–20 weeks old)

Mouse	n	Body wt g	Plasma Glucose mg/100 ml	Plasma Insulin ng/ml	Plasma Glucagon pg/ml
KK-C ♂	20	36.3±1.7	149.5± 7.4	4.52±1.79	320.0±60.6*
KK-CA^y ♂	15	50.1±1.3	304.3±24.2	21.42±3.48	786.3±42.2
KK-CA^y ♀	18	56.8±1.5	167.9±10.3	25.81±7.70	571.7±36.7

* mean±s.e.

elevated with the advance of age and the gain of body in both mice. The ratio of plasma IRI to blood glucose level in the male mice was distinctly lower than that in the female mice (Fig. 9). In the male severe hyperglycemics (over 300 mg/100 ml). little correlation was observed between both levels.

Plasma glucagon levels in male and female KK-CA^y mice

Table 1 shows simultaneously the determined levels of plasma glucose, insulin and glucagon in the KK-CA^y mice and their littermate control mice. As was expected, hyperglycemia was observed in the male KK-CA^y mice only, hyperinsulinemia in both KK-CA^y mice. Plasma glucagon level also were significantly elevated in the KK-CA^y mice compared with the control mice. Elevation was more marked in the male mice than the female mice.

Dose-dependent insulin-induced falling of blood glucose level in male KK-CA^y mice

The effect of insulin the on blood glucose level was assayed quantitatively in nonfasted KK-CA^y mice. Fig. 10–a shows the dose-dependent effect of insulin, representing falling percentage of the blood glucose level based on the value which was the mean of the nonfasted blood glucose level in control KK-C mice, 150 mg/100 ml. Fig. 10–b shows the dose-response curves for the percentage of blood glucose levels induced by insulin. One curve plotted by values of the maximal effect of each dose of insulin was little distinguishable from the other one plotted by the values of the effect 1 hr after application of insulin.

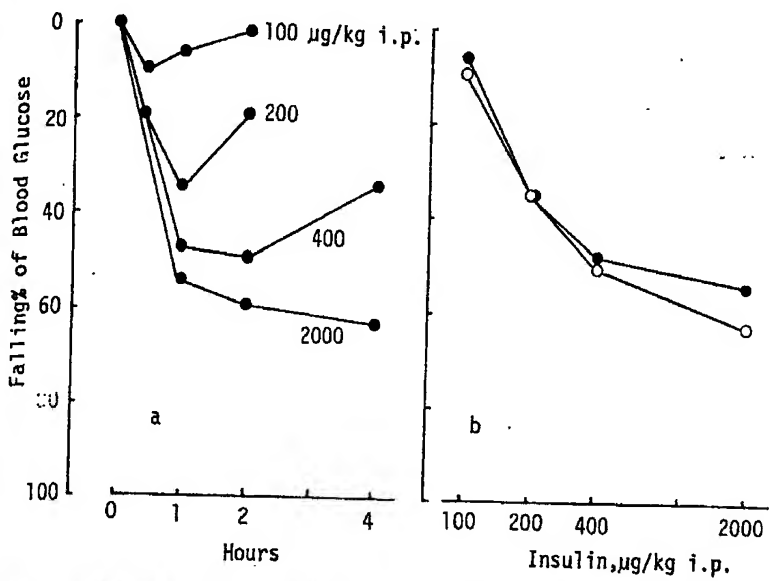

Fig. 10. Dose-dependent insulin-induced falling on blood glucose level in nonfasted KK-CA^y mice. a) Relationship between time and falling percentage of blood glucose level by the intraperitoneal application of insulin. b) Two dose-response curves for insulin application plotted by the values of the effect 1 hr after injection of insulin (●) and by the values of maximal effect of each dose of insulin (○).

Microscopic findings on pancreatic islets

On the pancreas of the KK-CA^y mice, morphological abnormalities were observed remarkably with the advance of age. Insular degranulation of B-cell was observed initially in a 6-week-old KK-CA^y mouse (Fig. 11–b). Insular hypertrophy also appeared in the pancreas of a 6-week-old KK-CA^y mouse and developed markedly in advancing age. In their control littermates, little changes were observed (Fig. 11–a). The changes mentioned above are summarized as the islet size in Table 2. In the female KK-CA^y mice, insular hypertrophy was observed without insular degranulation (Fig. 12). Furthermore, hepatocyte fatty degeneration, glomerular abnormalities, and proliferation of pituitary acidophil cell were also observed in the male KK-CA^y mice at 17 weeks of age. In their control littermates of the same age, these incidences were not or less observed. All microscopic findings were summarized in Table 3.

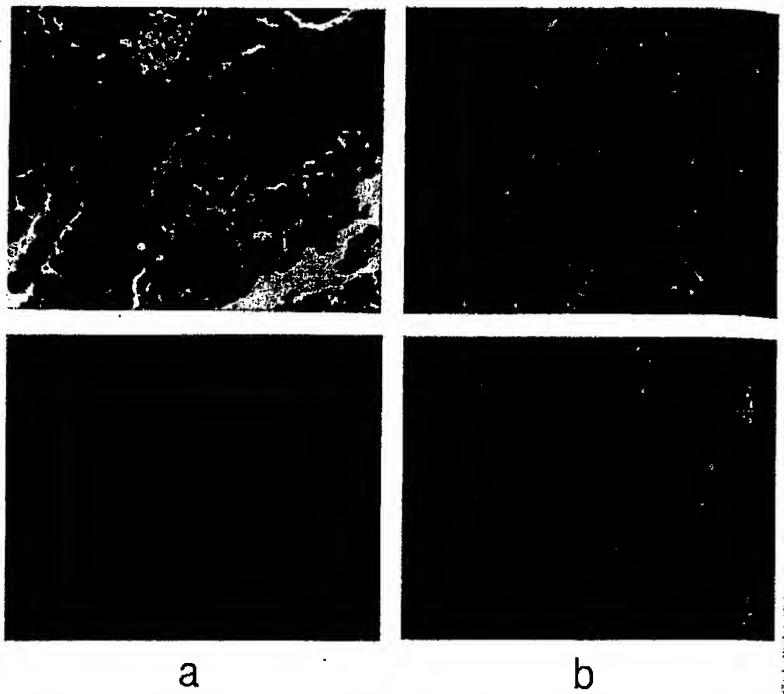

Fig. 11. Pancreatic islets of a control KK-C mouse (a) and a littermate KK-CA^y mouse (b) (male, 6 weeks old). Each section is stained with hematoxylin-eosin (above) and aldehyde-thionin (below), ×100.

Table 2. The size of pancreatic islets in KK-CA^y mice and their littermate KK-C mice

Mouse	age (weeks) 6	17	28	48
KK-C ♂	43±2 (73)	74±4 (60)	86±7 (28)	122±7 (60)*
KK-CA^y ♂	60±2 (130)	100±6 (55)	122±7 (39)	173±9 (78)

* mean±s.e. of islet size (μ) with the islet number (in parenthesis).
Individual islet size is the average diameter viewed under an optical microscope.

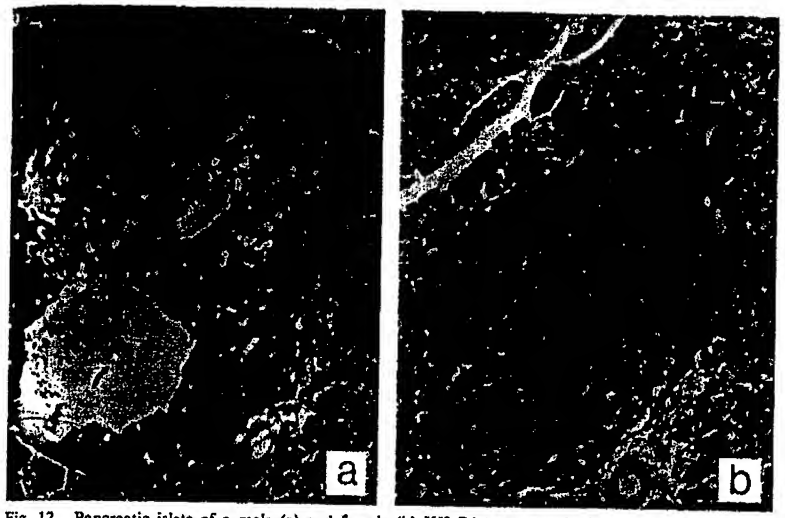

Fig. 12. Pancreatic islets of a male (a) and female (b) KK-CA^y mouse (28-29 weeks old). Pancreatic B-cells are stained by aldehyde-thionin stain, ×100. Insular degranulation and cavitation are observed only in the male mouse.

Table 3. Comparison of histological findings on pancreas and other organs from KK-CA^y mice and their control littermates

	Mouse[a]	KK-CA^y					KK-C				
	Age (weeks)	6	17	28	36	48	6	17	28	36	48
Histological findings											
Pancreas	insular hypertrophy	±	++	++	+++	+++	−	±	+	++	−[b]
	insular adhesion	−	−	−	++	++	−	−	−	−	−
	insular hemorrhage	−	−	+	++	+	−	−	−	−	−
	insular fibrosis	−	±	−	+	++	−	−	−	−	−
	sinusoidal dilatation	−	+	++	+++	++	−	−	−	++	+
	B-cell degranulation	+	++	++	+++	+++	−	−	±	++	+
	B-cell pleomorphism	−	+	++	+++	+++	−	−	−	++	+
Liver	hepatocyte fatty degeneration	±	++	++	+++	+++	−	−	+	+++	++
	hepatocyte vacuolation	−	+	++	+++	++	−	±	−	+++	++
	hepatocyte pleomorphism	−	+	++	++	++	−	±	±	++	+
Kidney	glomerular thickening	±	+	++	++	++	−	−	−	±	±
	glomerular hyaline deposits	−	±	++	+	+	−	−	−	−	−
	tubular hyaline casts	−	±	++	++	++	−	±	−	+	+
	interstitial cell infiltration	−	+	−	++	+	−	−	−	−	+
Adrenal	chromaffic cell vacuolation	−	−	±	++	+	−	±	−	−	+
Pituitary	acidophil cell proliferation	−	+	±	++	+	−	±	−	−	+

a) Males only were used.
b) A relative scale: (−) no histological change, (±) slight change, (+) moderate change, (++) marked change, (+++) strong change.

Discussion

It is generally agreed that a tendency to the development of human diabetes may be inherited with the complex genetic factors (Renold and Burr, 1970). As to the genetics of the KK mouse, Kondo *et al.* (1957) and Iwatsuka and Shino (1970) suggested that the inheritance is polygenic. The problem of genetic factors in the present study could be successfully avoided, because we used as a control the KK-C mice whose genetic factors were differed from those of KK-CA^y mice in only one gene (A^y).

In KK-CA^y mice the increase in blood glucose level was correlated with the increase in body weight, shown only in males. The female KK-CA^y mice showed considerably high degree of obesity, not severe hyperglycemia, indicating to be feasible to use as an obese model. In both sex mice the blood FFA levels increased more significantly than those in alloxan-induced diabetic mice. The increase in plasma triglyceride levels was also observed so that the abnormalities of fat metabolism might associate suggestively with the development of diabetes in this model. This suggestion is also supported by the report of Iwatsuka *et al.* (1970), that lipogenesis by liver and adipose tissue was increased in the "yellow KK" mice.

Hyperinsulinemia was often associated with obesity in humans (Ditschuneit, 1971) and animals (Stauffacher *et al.*, 1971). Bagdade *et al.* (1967) reported that in human obese subjects, but not diabetes, obesity was associated with an elevation of the basal insulin level and of the magnitude of the insulin response to glucose in oral glucose tolerance test whereas the response in diabetes, even in obese diabetics, was markedly reduced. Furthermore, the ratio of plasma insulin level to blood suger level (ΔIRI/ΔBS) was increasd in obesity, but not in diabetes with obesity (Kosaka *et al.*, 1977). The absolute concentration of plasma insulin does not always decrease in human diabetics. This point obviously differs from the alloxan-induced diabetic model. In the present study, the insulinogenic ratio of the control KK-C mice was not great any more than that of male KK-CA^y mice. Although KK-CA^y mice were hyper insulinemic compared with the control KK-C mice, they appeared to be deficient in insulin compared with female KK-CA^y mice (Table 1). The essential abnormalities of KK-CA^y mice seemed to be based on the relative decrease of plasma IRI level and to be distinguished from the obese factors (Fig. 9). This conclusion was supported by the histological evidence of the abnormalities of pancreatic islets (Fig. 11 and 12). These results indicate that the diagnosis of hyperinsulinemia may be misjudged by the selection of the control.

Recently, Unger *et al.* (1975) proposed that in addition to lack of insulin the presence of the insulin-opposing hormone glucagon is involved in the development of severe diabetic hyperglycemia. This evidence was also shown in spontaneous diabetic animals, for example, the obese hyperglycemic mice (ob/ob) (Mahler *et al.*, 1976). These suggestions were supported by our results (Table 1). The significance of the hyperglucagonemia in this model must be further elucidated, compared with that of the hyperinsulinemia.

In addition to that the diabetic features of the KK-CA^y mice seem to be similar to those of the diabetic patients, the dose-dependent response to insulin on blood glucose level, either the effect 1 hr after application or the maximal effect, was recognized in the case of nonfasting. This indicates the possibility of using the KK-CA^y mice as a feasible tool for a pharmacological assay of some antidiabetic drugs.

Acknowledgement

The authors are grateful to Dr. Z. Suzuoki and Dr. H. Iwatsuka, Biological Research Laboratory, Takeda Chemical Industries, for generous gift of breeder KK-CA^y mice.

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